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The nature of Kanagawa phenomenon (KH) of Vibrio parahaemolyticus was studied, and it was found that KH is an expression of strong hemolytic activity in media containing high concentration of NaCl, in which hemolytic factor diffused into media rapidly. Fermentable carbohydrates played an important role for the production of KH in media containing 7% NaCl, with different KH patterns by different carbohydrates. The promotion of growth by breakdown products of carbohydrates and lowered pH were supposed to influence the production of KH factor. (Cont)

In the study on survival of V. parahaemolyticus at various temperatures in sea water and 3% NaCl, this organism was found to grow at 37 C and 20 C. This organism showed a slow growth in 3% NaCl but not in sea water at 10 C or below. The survival time of this organism was 150 to 200 days in 3% NaCl at 37 C to 10 C, but was short and variable in sea water. Number of viable cells decreased rapidly at 4 C and below, but small numbers were cultured up to 5 to 15 days. The survival time varied by strains tested, and a tendency was suggested that strains of human origin survive longer than marine strains at 37 C with the adverse result at 10 C or below. (Author)

ABSTRACT

The nature of Kanagawa phenomenon (KH) of Vibrio parahaemolyticus was studied, and it was found that KH is an expression of strong hemolytic activity in media containing high concentration of NaCl, in which hemolytic factor diffused into media rapidly. Fermentable carbohydrates played an important role for the production of KH in media containing 7% NaCl, with different KH patterns by different carbohydrates. The promotion of growth by breakdown products of carbohydrates and lowered pH were supposed to influence the production of KH factor. In the study on survival of V. parahaemolyticus at various temperatures in sea water and 3% NaCl, the organism was found to grow at 37 C and 20 C. This organism showed a slow growth in 3% NaCl but not in sea water at 10C or below. The survival time of this organism was 150 to 200 days in 3% NaCl at 37 C to 10 C, but was short and variable in sea water. Number of viable cells decreased rapidly at 4 C and below, but small numbers were cultured up to 5 to 15 days. The survival time varied by strains tested, and a tendency was suggested that strains of human origin survive longer than marine strains at 37 C with the adverse result at 10 C or below.

TABLE OF CONTENTS

I.

Nature of the Kanagawa phenomenon of Vibrio parahaemolyticus.

1. Introduction.

The Kanagawa phenomenon of Vibrio parahaemolyticus, a clearly defined hemolysis on specially prepared media, was considered closely related to the enteropathogenicity (1), and several reports in Japan indicated that strains of V. parahaemolyticus isolated from stools of food poisoning patients are Kanagawa positive (K+), whereas almost all strains from marine specimens are Kanagawa negative (K-) (1,2). An heat-stable direct hemolysin was recently described as responsible for the Kanagawa type hemolysis (KH) (3). However, reports on food poisoning cases associated with K- strains are accumulating (4,5), and experimental results on the relationship between this hemolysis and the enteropathogenicity are contradictory, as reviewed by Twedt and Brown (6). We reported that KH is supposed to be quantitative rather than qualitative in nature, and to be an expression of strong hemolytic activity on special media (7). This report describes the effect of carbohydrates and other factors on KH.

2. Materials and methods.

Strains. Test strains listed in Table 1 were kindly supplied by R. Sakazaki, National Institute of Health, Japan, H. Zen-Yoji, Tokyo Metropolitan Research Laboratory, and Y. Miyamoto, Kanagawa Prefectural Public Health Laboratory, Japan. These included some K- strains isolated from stools of diarrheal patients. Other strains obtained from the above donors were used in an experiment.

Media. Modified Wagatsuma agar (Eiken, Japan) added with 5% human erythrocytes (WBA) was used for the determination of KH. In order to test various effects on KH, Wagatsuma base broth composed of 0.5% yeast extract, 1% peptone, 7% NaCl and 0.0001% crystal violet (pH 7.5) was prepared according to the formula of Wagatsuma agar. NaCl was always added to 3% in the other media used.

Determination of hemolytic activity. KH was determined according to the description of Miyamoto et al. (1). Briefly, cultures of test strains in nutrient broth (NB) were streaked linearly on WBA plates, and result was read after incubation for 24 hrs at 37 C. Well-defined clear hemolysis around the bacterial growth was considered as positive, no hemolysis as negative, and very narrow zone of hemolysis as doubtful. The observation of hemolysis was sometimes extended to 48 hrs. For the determination of diffused hemolysins into solid media, antibiotic assay cylinders placed on WBA plates were filled with extracts of media, and hemolysis around cylinders was observed after 24 hrs incubation at 37 C.

Determination of growth and pH. The growth of test strains in fluid media was determined by colony count on brom thymol blue teepol

Table 1. Origin and designation of *Vibrio parahaemolyticus*

Strain no.	Original designation	Kanagawa hemolysis	Source	Donor [a]
1	5507	+	Fish	Y. Miyamoto
3	8741	+	Responsible fish	Y. Miyamoto
5	9065	+	Responsible fish	Y. Miyamoto
8	9379	+	Patient's stool	Y. Miyamoto
10	9382	+	Patient's stool	Y. Miyamoto
12	9384	+	Patient's stool	Y. Miyamoto
23	T-3011	+	Patient's stool	H. Zen-Yoji
26	T-3031	+	Patient's stool	H. Zen-Yoji
51	9166	-	Patient's stool	Y. Miyamoto
54	9369	-	Patient's stool	Y. Miyamoto
56	8848	-	Fish	Y. Miyamoto
57	9331	-	Fish	Y. Miyamoto
61	70-3062	-	Fish	R. Sakazaki
62	70-3079	-	Fish	R. Sakazaki
66	T-3095-1	-	Patient's stool	H. Zen-Yoji
68	T-3232-1	-	Patient's stool	H. Zen-Yoji

[a] See text.

agar plates, or turbidometrically using B & L Spectronic 20 spectrophotometer at 540 nm. The pH was measured with Beckman Expandomatic SS-2 pH meter.

3. Results.

Table 2 shows the typical KH of strains obtained from Japan. Strains Nos. 1 through 30 were informed as K+ by the donors, and all showed positive reaction of varying degrees. Strains Nos. 51 through 79 were informed as K-, but three of them, Nos. 52, 58 and 76 were consistently K+ by repeated examinations. The hemolysis was intensified after 48 hrs, but the margin was not well-defined.

In order to test the activity of diffused hemolytic factors into agar underneath and around the bacterial growth, WBA and nutrient agar (NA) plates were inoculated with K+ and K- strains and incubated for 24 hrs. After swabbing the bacterial lawn, hemolytic zone of WBA and zone of bacterial growth of NA were cut and extracted by repeated freeze-thawing. The extracts were sterilized through Millipore filter (0.45 μ) and tested the activity. Figure 1 illustrates the hemolytic activity of WBA extracts. Two kinds of hemolytic zones, a clearly defined KH-like zone around the cylinder and an hazy zone around the clear zone, were produced by all extracts without qualitative difference between extracts from K+ and K- cultures. Only difference was that the extracts of WBA cultured with K+ strains produced larger hemolytic zones than those cultured with K- strains. One finding to mentioned is that the hemolysis underneath the bacterial growth of some K- strains at 24 hrs showed a colored map-like appearance with the mixture of clear and greenish red zones, while zone produced by K+ strains were clear.

Figure 2 illustrates the hemolysis of cultured NA extracts on WBA plates. Again, no qualitative difference was noted in hemolytic activities between agar extracts of K+ and K- strains, and larger zones were produced by extracts of K+ strains than by those of K-. The hemolytic pattern was the same with WBA extracts, but diameters of zones produced by NA extracts were much smaller than those produced by WBA extracts. These results suggest that hemolytic factors produced in WBA and NA by both K+ and K- strains are essentially the same. When the extracts were tested on ordinary blood agar plates, a narrow and hazy zone of hemolysis without clear margin was produced by both extracts of WBA and NA, and no difference was noted between extracts of K+ and K- strains.

The hemolysis of K+ and K- strains was observed on Wagatsuma base blood agar containing 0.5% carbohydrates to know the effect of carbohydrates on KH, and typical results are shown in Table 3. No hemolysis was produced by K+ and K- strains after 24 hrs at 37 C in media without carbohydrates or with non-fermentable sucrose and lactose, but KH-like hemolysis was noted at 24 hrs in the presence of fermentable carbohydrates, with different results by the strains and by carbohydrates added. The addition of maltose and trehalose in media turned more than half of K- strains into positive, and most

Table 2. Kanagawa type hemolysis of Vibrio parahaemolyticus

Strain[a] no.	Hemolysis 24[b]	Hemolysis 48	Strain no.	Hemolysis 24	Hemolysis 48
1	+[c]	+++[d]	51	-	-
2	±	+++	52	-	++
3	+	+++	53	+	+++
4	+	+++	54	-	+
5	+	+++	55	-	++
6	+	++	56	-	++
7	++	+++	57	-	+++
8	++	+++	58	+	+++
9	++	+++	59	-	++
10	++	+++	60	-	±
11	++	+++	61	-	+++
12	+	+++	62	-	++
13	+	+++	63	-	+
14	+	++	64	-	+
15	+	++	65	-	+
16	+	+++	66	-	+
17	++	+++	67	-	+
18	++	+++	68	-	+
19	++	+++	69	-	+
20	+	++	70	-	-
21	++	+++	71	-	++
22	++	+++	72	-	+
23	+	+++	73	-	+++
24	++	+++	74	-	++
25	++	+++	75	-	++
26	++	+++	76	++	+++
27	+	++	77	-	+
28	+	++	78	-	+
29	±	++	79	-	+
30	++	+++			

a Nos. 1-30 are informed as Kanagawa +, and 51-59 as Kanagawa -.

b Hours of observation.

c Homolytic zones: + = 1.5 mm or less, ++ = 1.5 - 3.0, +++ = 3.1 or more.

d Clear but hazy margin.

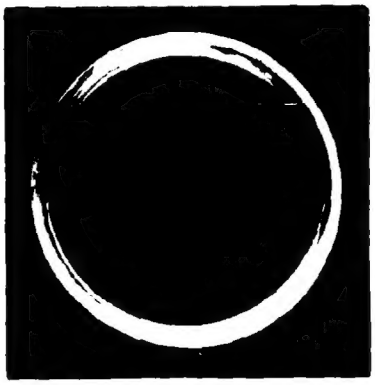

Figure 1. Hemolysis of Wagatsuma blood agar by extracts of Wagatsuma blood agar cultured with Kanagawa positive and negative strains of Vibrio parahaemolyticus. Upper left, strain No. 8 (K+); lower left, 12 (K+); upper right, 51 (K-); and lower right, 68 (K-). Dark zone around cylinder is clear hemolysis with distinct margin, and outer zone is hazy hemolysis without distinct margin.

Figure 2. Hemolysis of Wagatsuma blood agar by extracts of nutrient agar (3% NaCl) cultured with Kanagawa positive and negative strains of Vibrio parahaemolyticus. Upper left, strain No. 8 (K+); lower left, 12 (K+); upper right, 51 (K-); and lower right, 68 (K-). Dark zone around cylinder is clear hemolysis with distinct margin, and outer zone is hazy hemolysis without distinct margin.

Table 3. Effects of carbohydrates on Kanagawa type hemolysis[a] of Vibrio parahaemolyticus

Strain no.	None	Lactose	Sucrose	Dextrose[b]	Mannitol	Mannose	Maltose	Galactose	Trehalose	Arabinose
1	-[c]	-	-	+	+	+	+	-	-	±
3	-	-	-	++	+	+	+	-	+	-
5	-	-	-	+	+	+	+	-	+	-
8	-	-	-	+	+	+	+	-	+	-
10	-	-	-	+	+	+	+	±	+	-
12	-	-	-	++	+	+	+	-	+	±
23	-	-	-	++	+	±	±	-	-	-
26	-	-	-	++	+	+	+	±	+	±
51	-	-	-	+	-	±	±	-	+	-
54	-	-	-	+	-	-	-	-	+	-
56	-	-	-	+	-	-	+	-	+	-
57	-	-	-	+	-	±	+	+	+	-
61	-	-	-	+	-	+	-	-	+	-
62	-	-	-	+	-	+	-	-	-	-
66	-	-	-	+	-	±	+	-	+	+
68	-	-	-	+	-	-	+	±	-	+

[a] Wagatsuma base blood agar (see text) is incorporated with 0.5% carbohydrates.

[b] Bacterial growth is frequently surrounded by brownish turbidity.

[c] -, negative and +, positive, after 24 hrs.

K+ strains became negative in the presence of galactose and arabinose. The positive hemolysis of some K- strains was noted in mannose-containing media. The KH-like hemolysis of large zone was produced by both K+ and K- strains in dextrose-containing media, but the bacterial growth was frequently surrounded by brownish turbid zone.

Next studied was the effect of NaCl concentrations in media (Table 4). In case mannitol was incorporated, K+ strains produced clear KH-like hemolysis of large zone after 24 hrs in trypticase soy blood agar containing 7% NaCl, but K- strains did not. When NaCl was reduced to 3% in this medium, all K+ and a majority of K- strains showed clear but a narrow zone of hemolysis without distinct margin, and this is not likely to be KH. The KH-like hemolysis was also produced by K+ strains in NA containing blood and 7% NaCl, but not in NA containing blood and 3% NaCl where all K+ and most K- strains produced hemolysis of hazy margin. The inclusion of dextrose in media containing 7% NaCl produced clear KH-like hemolysis by both K+ and K- strains, but the brownish turbid zone was frequently noted around the bacterial growth. The hemolysis was different from KH in media with dextrose and 3% NaCl, and most strains showed a tendency of swarming growth. The swarming growth was also noted in WBA containing 3% NaCl. In an experiment, we noted that KH-like finding began to appear when NaCl concentration was 5% or more.

Three test strains; No. 5 which was K+ with mannitol and mannose; No. 54, K- with mannitol and mannose; and No. 61, K- with mannitol and K+ with mannose, were selected to study the relationship between KH and change of pH in media. Strains were inoculated on Nagatsuma base blood agar containing mannitol or mannose and incubated at 37 C. The bacterial growth was swabbed and agar underneath the bacterial lawn was cut. One gm of media was extracted in 9 ml of distilled water, and pH was determined in relation with the hemolysis (Table 5). K+ strains initiated KH from 15 hrs in the presence of mannitol, while K- strains showed hemolysis of hazy margin after 48 hrs. The pH of media decreased gradually during 18 hrs of incubation and then increased at 24 and 48 hrs, and the decrease was more marked in media grown with K+ strains than in media grown with K-. The addition of mannose in media produced similar results with mannitol-containing media, except for No. 5 by which pH of media decreased to 5.4 at 18 hrs with the concomitant brownish turbidity in media, especially in case media were heavily inoculated. The pH and the color change of media remained the same level during subsequent incubation. When media were inoculated lightly with No. 5, pH decreased to 6.0 after 18 hrs with positive KH.

Table 6 shows the growth of test strains and change of pH in 200 ml of Nagatsuma base broth without and with mannitol or mannose, inoculated with 0.1 ml of cultures in NB for 18 hrs. Only slight decrease of pH was observed during the incubation for 9 to 24 hrs in the absence of fermentable carbohydrates, with gradual decrease of light transmittance of media due to the bacterial growth. In the presence of mannitol or mannose, the bacterial growth was accelerated, and the marked decrease of pH was observed at 15 hrs of incubation

Table 4. Effect of NaCl concentrations on Kanagawa type hemolysis of Vibrio parahaemolyticus

Strain no.	TSA+7% NaCl[a]		TSA+3% NaCl		NA+7% NaCl[b]		NA+3% NaCl	
	Man[c]	Dex[d]	Man	Dex	Man	Dex	Man	Dex
1	K+[e]	K+	++[f]	++	K±	K+	+	+
3	K+	K+	++	++	K+	K+	+	+
5	K+	K+	++	++	K±	K+	+	+
8	K+	K+	++	++	K+	K+	+	+
10	K+	K+	+	++	K+	K+	+	+
12	K+	K+	+	++	K+	K+	+	+
23	K+	K+	+	++	K+	K+	+	+
26	K+	K+	+	++	K±	K+	±	+
51	K-	K+	+	++	K-	K+	±	+
54	K-	K+	±	+	K-	K+	±	±
56	K-	K+	+	++	K-	K±	+	+
57	K-	K+	+	++	K-	K+	+	+
61	K-	K+	-	+	K-	K+	+	+
62	K-	K+	+	++	K-	K+	+	++
66	K-	K+	+	+	K-	K+	+	+
68	K-	K+	+	++	K-	K+	±	+

a Trypticase soy agar with 5% erythrocytes and NaCl.
b Nutrient agar with 5% erythrocytes and NaCl.
c Mannitol.
d Dextrose.
e Kanagawa type hemolysis, + = positive, - = negative, at 24 hrs.
f Ordinary hemolysis (beta) without clear margin, ++ = strongly positive, at 24 hrs.

Table 5. Change of pH and Kanagawa type hemolysis of Vibrio parahaemolyticus on Wagatsuma blood agar

Carbohydrate added	Strain no.[a]	Hours of incubation						
		0	6	9	15	18	24	48
Mannitol	5	8.2[b]	7.7	7.2	6.0	5.7	5.8	8.7
		-[c]	-	-	±	+	+	+[d]
	54	8.2	7.9	7.6	7.0	7.0	7.4	8.7
		-	-	-	-	-	-	+
	61	8.2	7.7	6.9	7.4	7.4	7.8	8.8
		-	-	-	-	-	-	+
Mannoso	5[e]	8.2	6.8	6.4	5.5	5.4	5.4	5.4
		-	-	-	+	B[g]	B	B
	5[f]	8.2	7.5	7.0	6.2	6.0	6.9	8.8
		-	-	-	-	±	+	+
	54	8.2	7.9	7.7	6.3	6.8	7.9	8.7
		-	-	-	-	-	-	+
	61	8.2	7.3	6.6	5.8	6.3	6.9	8.6
		-	-	-	±	+	+	+

[a] No. 5 = Kanagawa + with mannitol and mannose, 51 = K- with mannitol and mannose, 61 = K- with mannitol and K+ with mannose.

[b] pH of agar.

[c] Kanagawa hemolysis, + = positive, - = negative, ± = doubtful.

[d] Margin of hemolysis become hazy after 48 hrs.

[e] Heavily inoculated.

[f] Lightly inoculated.

[g] Chocolate-like brownish turbidity in media.

Table 6. Growth of Vibrio parahaemolyticus and change of pH in Wagatsuma broth[a]

Carbohydrate added	Strain no.[b]	Test[c]	Hours of observation						
			0	6	9	15	18	24	48
None	5	pH	7.65	7.65	7.25	7.50	7.45	7.10	7.45
		T	100	92	77	69	65	57	60
	54	pH	7.55	7.55	7.55	7.00	6.95	6.85	7.40
		T	100	100	100	75	69	67	55
	61	pH	7.55	7.53	6.85	6.83	6.90	7.10	7.55
		T	100	88	61	59	59	57	47
Mannitol	5	pH	7.60	7.60	7.20	5.95	5.95	5.85	5.85
		T	100	89	57	44	44	44	44
	54	pH	7.60	7.60	7.60	6.90	6.30	6.00	5.70
		T	100	100	100	70	43	37	22
	61	pH	7.60	7.50	6.10	5.70	5.60	5.80	5.80
		T	100	88	47	34	35	28	18
Mannose	5	pH	7.55	7.55	7.25	5.50	5.45	5.43	5.35
		T	100	89	74	39	38	42	44
	51	pH	7.55	7.55	7.55	7.10	6.60	5.63	5.35
		T	100	95	94	76	64	52	49
	61	pH	7.50	7.30	6.20	5.35	5.32	5.40	5.40
		T	100	80	47	35	32	30	29

[a] Wagatsuma base broth (see text) with or without carbohydrates.

[b] No. 5 = Kanagawa + with mannitol and mannose, 54 = K- with mannitol and mannose, 61 = K- with mannitol and K+ with mannose.

[c] T = Percent transmittance of light at 540 nm.

with a little marked decrease in mannose-containing media than in mannitol-added media. Strain No. 54 seemed to show a little slow initial growth as compared with the other test strains.

The growth of K+ and K- strains was compared in fluid media (Table 7). Test strains showed a poorer growth in Wagatsuma base broth containing mannitol than in NB, and the increase of NaCl concentration to 8% resulted in the decrease of colony counts. The addition of erythrocytes in Wagatsuma base broth did not improve the growth.

The growth of V. parahaemolyticus was studied in buffered peptone water of varying pH (Table 8). All strains failed to grow at pH 5.2, and some strains did not even at pH 5.3 or 5.4. Again, there was no difference in pH limit of growth between K+ and K- strains.

Test strains were inoculated on WBA of different pH in order to know the effect of pH on the growth on solid media. WBA has a tendency of becoming brownish when pH was lowered to 5.4. No growth of inocula was noted at pH 5.3 and very scanty growth at pH 5.4. When erythrocytes were suspended in buffers of different pH containing 7% NaCl, only faint brownish hemolysis was observed after 24 hrs at pH 5.3 or lower, indicating that low pH only is not the cause of KH.

4. Discussion.

Even though KH of V. parahaemolyticus was reported to be a special hemolysis which is clearly identified on specially prepared media (1), our results indicated that the active factor was produced even in NA. Several investigators considered a thermostable direct hemolysin to be responsible for KH (3,8,9), and reported the purification of this factor from 15 to 18 hr-cultures of K+ strains but not from those of K- (8,9). K+ strains initiated K+ reaction from 15 to 18 hrs of incubation in WBA, and the short incubation time may be not enough for K- strains to produce sufficient KH factor to be determined. KH was clearly demonstrated in WBA but not in NA, and this result may be due to the difference of NaCl concentrations which would influence the diffusibility of the factor, as KH-like hemolysis was observed in nutrient and trypticase soy blood agars containing 7% but not in media containing 3% NaCl.

The presence of fermentable carbohydrates is indispensable for KH with different results by different carbohydrates. Our results suggest that the growth of V. parahaemolyticus is responsible for KH. WBA is a poor medium itself, possibly due to the high NaCl content, and breakdown products of carbohydrates were supposed to promote the growth. The acidic condition of media produced by the fermentation of carbohydrates may also favor the growth of some strains, as stated by Barrow and Miller (10). There is also a possibility that low pH may influence the production of KH factor, since KH was observed when pH of media was around 6.0 or lower.

Table 7. Growth of Vibrio parahaemolyticus in various media

Medium	Strain no.	Hours of observation				
		0	1	3	6	24
Nutrient broth 3% NaCl	1	565[b]	725	1,940	780,000	35,700,000
	51	175	370	1,870	1,010,000	30,800,000
Wagatsuma broth[a] 7% NaCl	1	320	255	585	13,400	5,900,000
	51	203	226	621	14,500	3,600,000
Wagatsuma broth 8% Nacl	1	181	277	305	1,950	1,950,000
	51	316	331	287	1,380	1,100,000
Wagatsuma broth 7% NaCl 5% RBC	1	543	485	436	12,400	3,200,000
	51	562	377	635	20,200	4,620,000

[a] Wagatsuma base broth with 0.5% mannitol and varying concn. of NaCl.

[b] Number of viable cells per 0.1 ml.

Table 8. Effect of pH on growth of *Vibrio parahaemolyticus* in NaCl yeast extract peptone water[a]

Strain no.	Inoculum (Fo/0.1ml)	5.4[b]	5.3	5.2
1	560	+[c]	+	-
3	520	++	+	-
5	270	++	++	-
8	1,040	+	+	-
10	1,380	+	-	-
12	950	-	-	-
23	1,210	-	-	-
26	175	±	-	-
51	1,880	++	+	-
54	690	+	-	-
56	870	±	-	-
57	390	++	++	-
61	2,110	+	+	-
62	1,900	+	-	-
66	2,200	+	±	-
68	140	+	±	-

[a] Strains are inoculated in a medium containing 3% NaCl, 0.5% yeast extract and 1% peptone in 0.01 M phosphate buffer (pH 8.5), and incubated for 24 hrs at 37 C.

[b] pH.

[c] Growth is determined turbidometrically at 540 nm. ++ = 79% or less in light transmittance (LT), + = 80 - 89% LT, ± = 90 - 99% LT, and - = 100% LT.

The result that Wagatsuma base blood agar with mannose showed sometimes brownish turbidity by the heavy growth of some K+ strains with lowered pH to 5.4, and the same change around the bacterial growth in the media containing dextrose, indicate that some carbohydrates lead to the lowered pH level at which brownish change occurs in media containing blood.

With those findings, we suppose that V. parahaemolyticus has different abilities to produce KH factor by strains, and the addition of fermentable carbohydrates may result in the promotion of growth with increased hemolysin production by the breakdown products in poor media containing 7% NaCl. The KH factor could diffuse rapidly in media of high NaCl content, and the quantitative difference of the KH factor would be demonstrated easily and clearly. There is a possibility that lowered pH and breakdown products of carbohydrates may play some roles on the production of the factor. Different carbohydrates produced different KH patterns. The relationship between KH and pathogenicity of V. parahaemolyticus was determined on empirical base (1), and it is not likely that the rapid hemolysin production in mannitol-containing media is only related to the enteropathogenicity of this organism. Our results of limited animal experiments showed no relationship between the pathogenicity and KH (7).

5. Summary.

Kanagawa type hemolysis (KH) of Vibrio parahaemolyticus was studied, and it was found that KH factor was produced in Wagatsuma blood agar and in nutrient agar containing 3% NaCl, and the difference in the KH was considered to be quantitative. The addition of fermentable carbohydrates is essential for KH with different KH patterns by different carbohydrates. KH was observed not only in Wagatsuma blood agar but also in nutrient and trypticase soy blood agars containing 7% NaCl, but not in media containing 3% NaCl. Fermentable carbohydrates promoted the growth of V. parahaemolyticus in poor media containing 7% NaCl and lowered pH of media.

Literature cited.

1. Miyamoto, Y., T. Kato, Y. Obara, S. Akiyama, K. Takizawa, and S. Yamai. 1969. In vitro hemolytic characteristics of Vibrio parahaemolyticus: Its close correlation with human pathogenicity. J. Bacteriol. 100:1147-1149.

2. Sakazaki, R., K. Tamura, T. Kato, Y. Obara, S. Yamai, and K. Hobo. 1968. Studies on the enteropathogenic, facultatively halophilic bacteria, Vibrio parahaemolyticus. III. Enteropathogenicity. Jap. J. Med. Sci. Biol. 21:325-331.

3. Yanagase, Y., K. Inoue, M. Ozaki, T. Ochi, T. Amano, and M. Chazono. 1970. Hemolysins and related enzymes of Vibrio parahaemolyticus. I. Identification and partial purification of enzymes. Bikon J. 13:77-92.

4. Molenda, J. D., W. G. Johnson, M Fishbein, B. Wentz, I. J. Mohlman, and T. A. Dasisman, Jr. 1972. Vibrio parahaemolyticus gastroenteritis in Maryland. Laboratory aspects. Appl. Microbiol. 24:444-448.

5. Terramoto, T., H. Nakanishi, K. Maeshima, and T. Miwatani. 1971. On the case of food poisoning caused by presumptive Kanagawa phenomenon negative strains (in Japanese). Media Circle 16: 174-177.

6. Twedt, R. M., and D. F. Brown. 1973. Vibrio parahaemolyticus: Infection or toxicosis. J. Milj Food Technol. 36:129-204.

7. Chun, D., J. K. Chung, and R. Tak. 1974. Some observations on Kanagawa type hemolysis of Vibrio parahaemolyticus, pp. 199-204. In Fujino, T., G. Sakaguchi, R. Sakazaki, and T. Takeda (ed.), Intern. Symp. on Vibrio parahaemolyticus (1973), Saikon Publ. Co., Tokyo, Japan.

8. Sakurai, J., A. Matsuzaki, and T. Miwatani. 1973. Purification and characterization of thermostable direct hemolysin of Vibrio parahaemolyticus. Infec. Immun. 8:775-780.

9. Sakurai, J., A. Matsuzaki, Y. Takeda, and T. Miwatani. 1974. Existence of two distinct hemolysins in Vibrio parahaemolyticus. Infec. Immun. 5:777-780.

10. Barrow, G. I., and D. C. Miller. 1974. Growth studies on Vibrio parahaemolyticus in relation to pathogenicity, pp. 205-210. In Fujino, T., G. Sakaguchi, R. Sakazaki, and T. Takeda (ed.), Intern. Symp. on Vibrio parahaemolyticus (1973), Saikon Publ. Co., Tokyo, Japan.

II.

Survival of Vibrio parahaemolyticus at various temperatures.

1. Introduction.

It is well known that the incidence of Vibrio parahaemolyticus in marine environments is closely correlated with water temperature, and the rise of the level of this organism in sea water appears to coincide with the spring-summer warming of estuarine and coastal sea water during April through October (1-4). This organism could not be detected in water during winter months although it was cultured from sea sediment, and the critical temperature for growth was reported to be around 10 C or higher in marine environments (1,4,5). This study was carried out to know the relationship between temperature and the survival of V. parahaemolyticus in sea water and NaCl solution.

2. Materials and methods.

Sea water was collected in winter from a beach, and was refrigerated for about two months to reduce marine bacteria. When V. parahaemolyticus was not detected, the sea water was used for experiments, even it contained a small number of organisms other than V. parahaemolyticus. The Cl^- ion concentration was 2.48% and pH was 8.5. Sea water was divided into four parts; one part was non-treated, and the others were treated as follows: filtration through Chamberland L3 filter, boiling for 30 minutes, and sonication for 20 minutes with Branson Biosonic II oscillator. Treated and non-treated sea water and 3% NaCl in double distilled water were dispensed in large tubes.

Four strains of V. parahaemolyticus were used for the study. Two strains Nos. P3 (K3) and P11 (K55) were isolated from stools of diarrheal patients in this laboratory, and the other two, Nos. 444 (K34) and 445 (K42) were isolated from sea fish. An equal amount of cultures of test strains in nutrient broth containing 3% NaCl was mixed, diluted to 1,000 times with 3% NaCl, and 0.4 ml were inoculated in 50 ml of sea water and 3% NaCl. Viable cells of each test strains inoculated were 900 to 3,100 per 0.1 ml of test media. The test media were then being held at 37 C, 20 C, 10 C, 4 C, -10 C and -20 C, and shaking was avoided during the incubation, except for the time of counting viable cells. The survival of organisms was observed by colony count in 0.1 ml of media on brom thymol blue teepol agar plates at various intervals.

3. Results.

Table 1 shows the survival of test organisms at 37 C. The number of organisms increased significantly during incubation for one to two days in all media, especially in 3% NaCl, and then decreased gradually following incubation. Organisms were cultured in considerably large numbers even after 100 days in 3% NaCl, and in about an half numbers of inoculated at 50 days in filtered sea water. The survival in

Table 1. Survival of Vibrio parahaemolyticus in sea water and 3% NaCl at 37 C

Days of observ.	Treatment of sea water				3% NaCl
	None	Filtered	Boiled	Sonicated	
0	7,600[a]	4,900	8.100	5,000	5,000
1	232,000	84,000	70,000	131,000	879,000
2	400,000	210,000	230,000	256,000	668,000
3	114,000	82,000	125,000	85,000	525,000
5	23,000	48,000	93,000	16,000	382,000
10	5,400	45,000	53,000	28,000	150,000
15	3,200	16,000	1,230	830	90,000
20	670	9,000	184	79	82,000
30	194	9,700	135	64	81,000
40[b]	37	4,900	74[b]	77	46,000
50	29[b]	2,180[b]	80	54[b]	27,000[b]
60	0	1,290	0	27	7,200
70	0	870	0	16	5,300
80	0	810	0	11	3,200
90	0	480	0	0	2,100
100	0	106	0	0	1,600
150	0	0	0	0	45
200	0	0	0	0	0

[a] A mixture of broth cultures of test strains was diluted, inoculated in test media, and number of viable cells in 0.1 ml was counted.

[b] Colonies were serotyped (see text).

non-treated, boiled and sonicated sea water was shorter than in filtered sea water, and organisms were not cultures after 60 to 90 days. At 40 to 50 days of incubation, colonies selected at random were serotyped, all from non-treated sea water and 3% NaCl were typed as P3. The ratios of P3 and 444 in filtered, boiled and sonicated sea water were 19:1, 3:1 and 4:1, respectively, and no colonies were typed into P11 and 445.

When test media were being held at 20 C, the increase in number of organisms was observed in 2 to 5 days and followed by gradual decrease (Table 2). Large numbers of viable cells were counted in 3% NaCl even after 250 days, and it seemed that this organism was stabilized by prolonged incubation in 3% NaCl at this temperature. Only small numbers of V. parahaemolyticus survived up to 40 to 70 days in treated and non-treated sea water, with a little longer survival in filtered sea water. Colonies cultured from sea water at 20 to 50 days and those from 3% NaCl at 100 days were predominatly 444, and only a small numbers of P3 were typed. No colony belonging to P11 and 445 was typed.

The survival of V. parahaemolyticus at 10 C is shown in Table 3. Number of viable cells increased gradually with maximum numbers at 5 to 20 days in 3% NaCl, and persisted in small numbers for more than 200 days. In sea water, viable cells persisted 5 to 30 days without initial increase in numbers, with a little longer persistence in filtered sea water than in others. Colonies were typed at 5 to 50 days and all were classified into 444.

Table 4 shows the survival of test organisms at 4 C, -10 C and -20 C. Inoculated organisms were inactivated within 8 to 20 days of preservation, and the inactivation occurred more rapidly in 3% NaCl than in others. Organisms survived for 5 to 15 days in sea water were exclusively 444.

4. Discussion.

Results in this study indicated that V. parahaemolyticus can grow in 3% NaCl, and this is in agreement with the results of Chun et al. (6,7) who reported the growth of this organism in phosphate-buffered saline. Since broth cultures used as inocula were finally diluted more than 100,000 times in test media, ingredients of broth contained in inocula seemed not to play a significant role for growth, and the nutritional requirement of this organism is supposed to be very simple. Three percent NaCl allowed the survival of this organism for more than 150 days at 10 C and above, but sea water allowed the shorter survival than 3% NaCl. Sea water was collected at a beach and it may have been contaminated with human waste drained from nearby communities and from ships, and these contaminants may play an adverse role for the survival of V. parahaemolyticus. There is also a possibility that ingredients of sea water other than NaCl may play some role in the shortened survival. Ingredients of sea water will vary according to the place of collection, and some sea water will be more favorable than others for growth of this organism. Treatment

Table 2. Survival of Vibrio parahaemolyticus in sea water and 3% NaCl at 20 C

Days of observ.	Treatment of sea water				3% NaCl
	None	Filtered	Boiled	Sonicated	
0	7,600	4,900	8,100	5,000	5,000
1	17,600	10,200	12,300	28,000	230,000
2	36,000	11,000	146,000	27,000	840,000
3	1,700	6,200	7,400	36,000	1,180,000
5	280	5,500	1,500	25,100	1,030,000
10	38	4,200	640	8,100	925,000
20	5[a]	4,100	147[a]	350	365,000
30	2	3,700	35	43	293,000
40	1	1,200	30	38[a]	186,000
50	0	660[a]	0	2	113,000
60	0	85	0	0	81,000
70	0	1	0	0	72,000
80	0	0	0	0	45,000
90	0	0	0	0	46,000
100	0	0	0	0	45,000[a]
150	0	0	0	0	44,300[a]
200	0	0	0	0	37,900
250	0	0	0	0	36,100

[a] Colonies were serotyped (see text).

Table 3. Survival of Vibrio parahaemolyticus in sea water and 3% NaCl at 10 C

Days of observ.	Treatment of sea water				3% NaCl
	None	Filtered	Boiled	Sonicated	
0	7,600	4,900	8,100	5,000	5,000
1	7,400	2,200	4,800	4,100	18,000
2	3,400	3,700	17,700	3,500	40,000
3	1,320	3,300	4,300	3,200	86,000
5	50[a]	2,400	810	3,700	197,000
8	0	1,500	140	1,600	215,000
10	0	1,100	65[a]	565[a]	254,000
20	0	150[a]	2	0	210,000
30	0	110	0	0	59,000
40	0	0	0	0	28,000
50	0	0	0	0	18,400[a]
60	0	0	0	0	9,100
70	0	0	0	0	2,900
80	0	0	0	0	700
90	0	0	0	0	650
100	0	0	0	0	330
150	0	0	0	0	112
200	0	0	0	0	103

[a] Colonies were serotyped (see text).

Table 4. Survival of Vibrio parahaemolyticus in sea water and 3% NaCl

Temperature	Days of observ.	Treatment of sea water				3% NaCl
		None	Filtered	Boiled	Sonicated	
4 C	0	7,600	4,900	8,100	5,000	5,000
	1	3,800	1,300	2,600	1,500	2,000
	2	1,300	1,700	4,300	1,600	1,100
	3	1,540	600	1,530	420	100[a]
	5	80[a]	330	940	310	40[a]
	8	0	90[a]	770	63	12
	10	0	7	550	21[a]	0
	15	0	0	19[a]	0	0
	20	0	0	0	0	0
-10 C	0	7,600	4,900	8,100	5,000	7,400
	1	3,600	560	660	800	460
	2	2,300	550	420	850	4
	3	720	180	350	340	1
	5	152	40	83	175	0
	8	0	38[a]	58	142	0
	10	0	5[a]	11[a]	23[a]	0
	15	0	0	1	1	0
	20	0	0	0	0	0
-20 C	0	7,600	4,900	8,100	5,000	7,400
	1	2,900	1,170	990	340	4
	2	1,720	418	186	339	3
	3	530	113	71	105	1
	5	114	15	60	49	0
	8	65	26	44	16[a]	0
	10	35[a]	18[a]	3[a]	2	0
	15	0	0	0	0	0

[a] Colonies were serotyped (see text).

of sea water brought about different results on the survival, and the reasons were not studied in this report. The longest survival of V. parahaemolyticus in filtered than in other sea water may be related to the filtration and elimination of marine contaminants which played an adverse role for the survival of this organism. Our results on the survival of V. parahaemolyticus for 30 days at 10 C in filtered sea water and more than 200 days in 3% NaCl suggest a possibility of over-wintering in sea water of favorable conditions for survival in the temperate zone where water temperature is around 10 C or above in winter. No noticeable growth was noted at 10 C, and this result is in agreement with the reports that the critical temperature for growth is about 10 C or above (1,4,8).

Number of viable cells decreased without initial growth at 4 C or below, with survival up to 5 to 15 days. Johnson et al. (9) reported the survival of V. parahaemolyticus at least 3 weeks in oyster shellstock at refrigeration temperature, and it seems that the refrigeration for short periods would not eliminate the possibility of food poisoning due to sea food. Since this organism was rapidly inactivated in distilled water (10) and in tap water (Chun, unpublished data), thorough washing of sea food with tap water is supposed to be an important procedure for the prevention of food poisoning.

We noted the difference in the survival time by strains. We also found that one strain of human origin survived longer than a marine strain at 37 C and lowered temperature resulted in the prolonged survival of a marine strain. These results suggest that the incidence of food poisoning due to V. parahaemolyticus may be related, at least in some aspects, with the prolonged survial of this organism around 37 C.

5. Summary.

Vibrio parahaemolyticus propagated at 37 C and 20 C in sea water and 3% NaCl, and survived as long as 150 to 200 days in 3% NaCl. In sea water, the survival was shorter than in 3% NaCl, with varying survival time by the treatments of sea water. V. parahaemolyticus did not grow in sea water at 10 C, but showed a slow growth in 3% NaCl. The survival at 10 C was more than 200 days in 3% NaCl and 5 to 30 days in sea water. Number organisms decreased rapidly at 4 C or below, with survival of 5 to 15 days.

Literature cited.

1. Noguchi, M. 1963. Ecological investigation (in Japanese). pp. 289-311. In Fujino, T., and H. Fukumi (ed.), Vibrio parahaemolyticus I. Itscido, Tokyo, Japan.

2. Baross, J., and J. Liston. 1970. Occurrence of Vibrio parahaemolyticus and related hemolytic vibrios in marine environments of Washington State. Appl. Microbiol. 20:179-186.

3. Koburger, J. A., and C. R. Lazarus. 1974. Isolation of Vibrio parahaemolyticus from Salt Springs in Florida. Appl. Microbiol. 27:435-436.

4. Kaneko, T., and R. R. Colwell. 1973. Ecology of Vibrio parahaemolyticus in Chesapeake Bay. J. Bacteriol. 113:24-32.

5. Chun, D., J. K. Chung, S. Y. Seol, and R. Tak. 1974. Vibrio parahaemolyticus in the Republic of Korea. Amer. J. Trop. Med. Hyg. 64:1125-1130, in press.

6. Chun, D., S. Y. Seol, R. Tak, and C. K. Park. 1972. Inhibitory effect of glycerin on Vibrio parahaemolyticus and Salmonella. Appl. Microbiol. 24:675-678.

7. Chun, D., J. K. Chung, and S. Y. Seol. 1974. Enrichment of Vibrio parahaemolyticus in a simple medium. Appl. Microbiol. 27:1124-1126.

8. Bradshaw, J. G., D. W. Francis, and R. M. Twedt. 1974. Survival of Vibrio parahaemolyticus in cooked seafood at refrigeration temperatures. Appl. Microbiol. 27:657-661.

9. Johnson, Jr., W. G., A. C. Salinger, and W. C. King. 1973. Survival of Vibrio parahaemolyticus in oyster shellstock at two different temperatures. Appl. Microbiol. 26:122-123.

10. Lee, J. S. 1972. Inactivation of Vibrio parahaemolyticus in distilled water. Appl. Microbiol. 23:166-167.

III.

List of publications.

1. Chun, D., S. Y. Seol, R. Tak, and C. K. Park. 1972. Inhibitory effect of glycerin on Vibrio parahaemolyticus and Salmonella. Appl. Microbiol. 24:675-678.

2. Kim, B. I., J. K. Chung, and D. Chun. 1973. Effect of Korean seasonings on Vibrio parahaemolyticus (in Korean and summary in English). Korean Cent. J. Med. 24:25-32.

3. Chun, D., J. K. Chung, and R. Tak. 1974. Some observations on Kanagawa type hemolysis of Vibrio parahaemolyticus. Intern. Symp. on Vibrio parahaemolyticus, pp. 199-204, Saikon Publ. Co., Tokyo, Japan.

4. Chun, D., J. K. Chung, and S. Y. Seol. 1974. Enrichment of Vibrio parahaemolyticus in a simple medium. Appl. Microbiol. 27:1124-1126.

5. Chun, D., J. K. Chung, S. Y. Seol, and R. Tak. 1974. Vibrio parahaemolyticus in the Republic of Korea. Amer. J. Trop. Med. Hyg. 64:1125-1130, in press.